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Fungi Resident in Chickpea Debris and their Suppression of Growth and Reproduction of *Didymella rabiei* under Laboratory Conditions

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Abstract

Fungi colonizing senescent chickpea (*Cicer arietinum*) stems and postharvest debris from Pullman, WA, were enumerated and identified with the objective of finding species potentially useful for biological control of *Didymella rabiei* (conidial state = *Ascochyta rabiei*), causal agent of Ascochyta blight. In addition to *D. rabiei*, primary colonizers were, in order of decreasing abundance, *Alternaria tenuissima*, *Al. infectoria*, *Ulocladium consortiale*, *Epicoccum purpurascens*, *U. atrum* and *Fusarium pseudograminearum*. Present at lower frequencies were *Al. malorum*, *Cladosporium herbarum*, *Aureobasidium pullulans*, *Clonostachys rosea* and miscellaneous anamorphic ascomycetes. On agar media and autoclaved chickpea stems, *Au. pullulans* consistently grew faster than *As. rabiei*, and excluded *As. rabiei* from the substrate. When stems received prior inoculation with *Au. pullulans* or *Cl. rosea*, followed by inoculation with compatible mating types of *D. rabiei*, formation of pseudothecia and pycnidia of *D. rabiei* was suppressed. Results suggest that *Au. pullulans* and *Cl. rosea* can inhibit *As. rabiei* and its sexual stage, *D. rabiei*, on chickpea debris. *Clonostachys rosea* formed appressoria on, then invaded, hyphae of *D. rabiei*. Small-scale field experiments using *Au. pullulans* and *Cl. rosea* have been initiated.

Introduction

The adoption of reduced tillage practices to control soil erosion in US Pacific Northwest (PNW) cropping systems has created conditions that are ideal for the survival and reproduction of *Ascochyta rabiei* (Pass.) Lab., the causal agent of Ascochyta blight of chickpea (*Cicer arietinum* L.). The sexual stage, *Didymella rabiei* (Kovachevski) Arx, develops during the winter on chickpea debris, and ascospores infect newly emerging chickpeas the following season (Kaiser, 1992). One of the best control measures for Ascochyta blight is the deep ploughing of infected plant residues (Kaiser and Hannan, 1987); however, this practice is incompatible with soil conservation practices. The increasing amount of plant debris left on the soil surface is thought to have led to a large increase in inoculum for Ascochyta blight epidemics and to the increased severity of blight epidemics observed in the PNW the past few years. Given the importance of soil erosion control and the inevitable move towards reduced tillage in PNW agriculture, control of Ascochyta blight will increasingly depend on suppressing the sexual stage of the pathogen.

The primary type of inoculum for Ascochyta blight epidemics in the PNW is thought to be ascospores (Trapero-Casas and Kaiser, 1992a,b; Trapero-Casas et al., 1996). This is based both on low rates of seed infection of PNW seed lots, and anecdotal observations of disease pattern and timing in the field (T. L. Peever, W. Chen and W. J. Kaiser, unpublished data) as well as on indirect evidence from mating type ratios and multilocus gametic disequilibrium tests with genetic markers which indicate a recombined population structure (Peever et al., 2004). Ascospores land on chickpea plants, infect directly and form lesions, which produce pycnidia. Secondary spread of Ascochyta blight within chickpea fields during the growing season is dependent upon rainfall events, which disperse conidia to uninfected plants and create appropriate environmental conditions for infection (Trapero-Casas and Kaiser, 1992a,b). The sexual stage is formed on chickpea debris colonized by the fungus the previous season and requires approximately 2 months at cool temperatures (5–10°C) to mature and produce ascospores. Ascospores are released under specific environmental conditions, are wind-borne, and can infect chickpea plants several hundred metres or more from the source, while conidia are splash-dispersed only short distances (Kaiser, 1992; Trapero-Casas et al., 1996). Long-distance movement of ascospores is the

most likely route by which the pathogen is moved among chickpea fields and from infected to uninfected fields. Our objectives here were to identify the primary fungal competitors of *D. rabiei* in colonization of chickpea debris, and to conduct preliminary tests with one or more of these competitors to assess the ability of these fungi to inhibit growth and reproduction of *D. rabiei*.

Materials and Methods

Floristic analysis of fungi colonizing chickpea debris

Chickpea stems were collected on 27 August 2003 at Washington State University's Spillman Research Farm, Pullman, Washington and 9 September 2004 at a commercial farm near Genesee, Idaho. Stems from the Washington site were collected at harvest; those from Idaho approximately 2 weeks after harvest. For each location, segments 2 cm long were excised from 100 stems. Prior to transfer to standard culture dishes containing agar media, 25 of the segments were washed in running tap water for 2 h, 25 were disinfested by immersion for 10 s in 70% ethanol then rinsed with sterile H_2O, and 50 were disinfested by immersion for 1 min in 0.5% NaOCl then rinsed with sterile H_2O. Approximately one-third of the stem segments from each of the two locations and from each of the disinfestation treatments were plated on malt agar amended with rose bengal (Dugan and Lupien, 2002) and approximately two-thirds were plated to half strength V8 agar (1/2V8; Stevens, 1981). Each of the agar media was amended with 50 μg/ml each of streptomycin sulphate and tetracycline hydrochloride (Sigma®, St Louis, MO, USA). Cultures were incubated under ambient laboratory conditions (approximately 22°C, daytime fluorescent light), during which time fungal isolates were subcultured to slants of 1/2V8 and incubated under near ultraviolet and fluorescent light (12 h/12 h light–dark cycle) at ambient temperature for recovery of fungi. Isolates recovered were identified by published morphological and physiological criteria (Simmons, 1967, 1986, 1990, 1995; Ellis, 1971, 1976; Kushwaha and Agrawal, 1976; Hermanides-Nijhof, 1977; Nelson et al., 1983; dos Santos et al., 1993; de Hoog and Yurlova, 1994; Andersen and Thrane, 1996; Seifert, 1996; Aoki and O'Donnell, 1999; Ho et al., 1999; Yurlova et al., 1999; Schroers, 2001). Taxa which were recovered in highest abundance (*Alternaria*, *Ulocladium*), were subsampled at random by generation of single-spore isolates for every third isolate (for *Ulocladium*) to sixth isolate (for *Alternaria*).

Relative growth rates and interspecies growth inhibition

Didymella rabiei, Aureobasidium pullulans and Clonostachys rosea Strains of *D. rabiei*, *Au. pullulans* and *Cl. rosea* isolated from chickpea are listed in Table 1.

Relative growth on chickpea stems For assessing relative growth of *As. rabiei* (isolates AR19, AR628) and *Au. pullulans* (AuP12, AuP26), incubation chambers were constructed by fixing autoclaved segments of chickpea stems to glass rods with drops of silicone adhesive, and placing the rods and stems on a stainless steel disc over a pool of sterile distilled water (higher humidity) or a mixture of 80% sterile distilled water and 20% glycerol (lower humidity) within a 15-cm-diameter glass culture dish (Fig. 1). Within each chamber, four groups of two stems each were inoculated at stem midpoint with a single isolate of one species or the other, and growth rates were measured and averaged for each stem so that average growth rates were twice obtained for two isolates of *As. rabiei* and two isolates of *Au. pullulans* within a single chamber. There were two chambers for each level of relative humidity (RH) and the design was blocked by chamber for statistical analysis. Chambers were sealed with Parafilm® (Menasha, WI, USA) and incubated under fluorescent and near ultraviolet light (12 h light/12 h dark) at 15°C. Relative humidity was monitored by construction of two additional chambers, each enclosing a Humidity-On-A-Card™ (Houston, TX, USA). The entire experiment was repeated at 20°C. An analogous set of chambers using *As. rabiei* (AR19, A628) and *Cl. rosea* (CP63E, CP98B) was constructed and incubated at 20°C with RH ≥90%. In all instances, stem ends were excised from stems at completion of the experiment, and transferred to 1/2V8 for confirmation of identity of the fungi and to check for contamination.

Table 1
Strains used for experimentation

Ascochyta rabiei (single-spore isolates)	
AR19 (ATCC 24891); Iran, December 1990; W. J. Kaiser	
AR20 (ATCC 76501); Idaho, USA, May 1986; W. J. Kaiser	
AR21 (ATCC 76502); Idaho, USA, June 1986; W. J. Kaiser	
AR428; Idaho, USA, 1994; W. J. Kaiser	
AR460; Washington, USA, 1984; W. J. Kaiser	
AR462; Washington, USA, 1983; W. J. Kaiser	
AR628 (AA13 from ICARDA), Syria, June 1995; W. J. Kaiser	
AR630; Washington, USA, July 1995; W. J. Kaiser	
Aureobasidium pullulans (single-spore isolates)	
AuP12, chickpea debris; Idaho, USA, June 2002; F. M. Dugan	
AuP26, chickpea debris; Idaho, USA, June 2002; F. M. Dugan	
Clonostachys rosea (single-spore isolates)	
CP63E, chickpea debris; Washington, USA, 27 August 2003; W. Chen	
CP98B, chickpea debris; Washington, USA, 27 August 2003; W. Chen	
Single-spore strains, chickpea debris; Washington, USA, August 2003; W. Chen	
Alternaria tenuissima	CP7C
	CP22B
Alternaria infectoria	CP11A
	CP93A
Ulocladium consortiale	CP1B
	CP78B
Epiccocum purpurascens	CP74H
	CP83A
Stemphylium sp. 1	Tobin1
	Tobin2

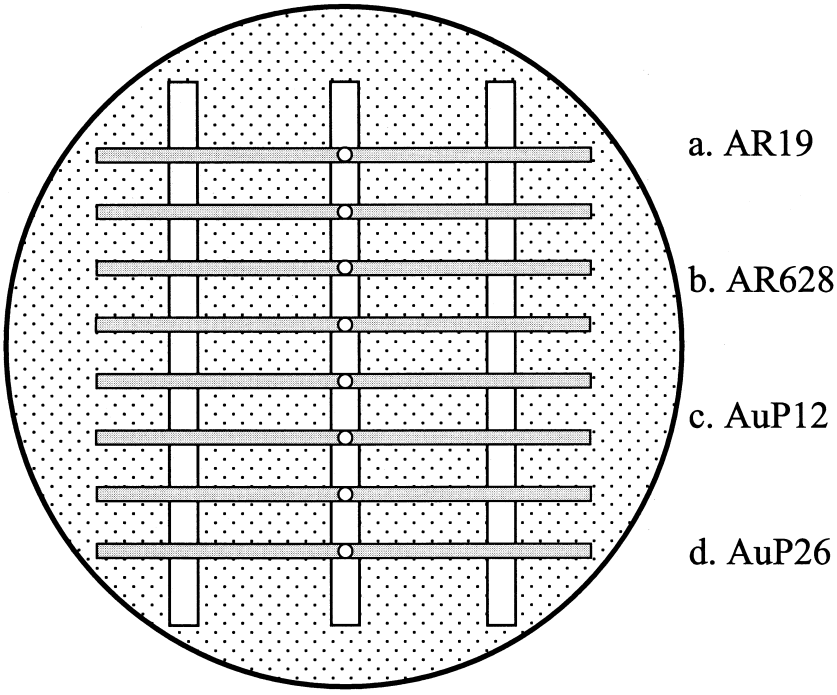

Fig. 1 Diagram of typical chamber for measuring growth along chickpea stems. Two stems were used per strain with two strains of *Ascochyta rabiei* (stem sets a and b) and two of *Aureobasidium pullulans* (stem sets c and d) in each chamber. Small circles at midstem represent inoculation sites

Interspecies growth inhibition of As. rabiei and Au. pullulans on chickpea stems Another set of chambers (Fig. 2) was produced and inoculated on the same date as the experiment above. Within a single chamber, four groups of two stems each were inoculated at stem midpoint with *As. rabiei* (either AR19 or AR628), and at both stem ends with *Au. pullulans* (either AuP12 or AuP26), such that, within a single chamber, each AR isolate was paired once with each AuP isolate, and the colonies were allowed to grow towards one another. There were two replicate chambers for each combination of temperature (15°C or 20°C) and RH (RH ≥ 80% or RH ≥ 90%). Colony growth rate and other characters were monitored as colonies converged. Reactions (colour, presence of aerial hyphae and/or fruiting bodies) were noted every 2 days for each colony on each stem at 10–60×. About 2–4 days after the slowest growing isolate of *As. rabiei* had reached ends of the stems above (Fig. 1), approximately 5 mm from ends of the stems in the chambers designed to test inhibition (Fig. 2) were harvested and placed onto 1/2V8 and incubated at laboratory conditions as above for any recovery of *As. rabiei* from the ends occupied by *Au. pullulans* colonies. The assumption was that the stems inoculated only at midpoint with *As. rabiei* (Fig. 1) would give a measure of the time necessary for uninhibited *As. rabiei* to grow to the ends of the stems, so that subsequent failure to recover *As. rabiei* from the distal ends of corresponding stems illustrated in Fig. 2 would constitute further evidence of the ability of *Au. pullulans* to exclude *As. rabiei* from the substrate. Data from each experiment were subjected to PROC ANOVA and/or PROC GLM in SAS 8.2 (SAS Institute, Inc., Cary, NC, USA) and/or ANOVA in SYSTAT 9.0 (SPSS Science, Chicago, IL, USA).

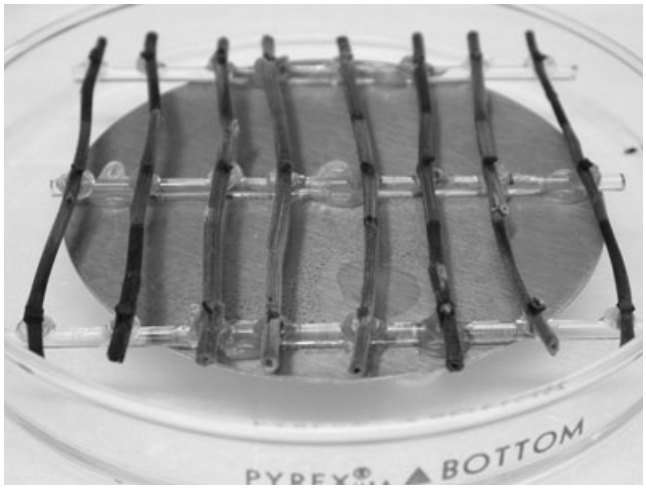

Fig. 2 Chamber for monitoring interspecific inhibition on chickpea stems. Stems were inoculated at midpoint with *Didymella rabiei* and at both ends with *Aureobasidium pullulans*

Inhibition of formation of ascospores and conidia by preinoculation of substrate with *Au. pullulans*

Stem sections (approximately 3.5 cm each) were excised from healthy dried chickpea plants, autoclaved twice on each of 2 successive days for 30 min at 121°C while suspended over 2.5 cm of water in glass beakers. Multiple pairs of isolates of *As. rabiei* of compatible mating types were employed to initiate crosses on stems that were preinoculated with *Au. pullulans*. Sets of controls were: (i) stems not preinoculated with *Au. pullulans* and inoculated with compatible mating types of *As. rabiei*; (ii) two self-crosses as negative

controls for comparison with outcrosses. *Didymella rabiei* is heterothallic with a bipolar mating system (Wilson and Kaiser, 1995; Barve et al., 2003). Crosses are shown in Table 2.

Preinoculation Inoculum of *Au. pullulans* isolate AuP12 was increased by streaking ontoV8 medium and incubating at 22–25°C under combined cool white and near UV light on an alternating 12-h photoperiod for 6 days. Conidia were harvested by washing the agar surface of cultures with sterile deionized water and concentration was adjusted to 6.25×10^6 conidia/ml. Autoclaved chickpea stems were immersed in conidial suspension for 2.5 h with intermittent mild agitation. Stems were transferred to chambers (sterile glass Petri dishes with 15 layers of sterile Whatman No. 1 filter paper with 10 ml sterile deionized water) and incubated unsealed for 6 days at 22–25°C during which an additional 4 ml of sterile water was added to each chamber.

Inhibition of ascospore production Production of ascospores was from Trapero-Casas and Kaiser (1992b) and Wilson and Kaiser (1995) with slight modification. Nine crosses were performed between six mating-compatible monosporic isolates (*MAT1-1* or *MAT1-2*; Table 2). Crosses were repeated using chickpea stems that had been preinoculated with AuP12. Following the incubation period, chickpea stems were suspended over 3% water agar plates, allowing ascospores from compatible crosses to be released onto the agar surface below. Ascospores were quantified by examination of the agar surface at 50–100×. Suppression of the asexual state was monitored by observation of development of pycnidia on controls relative to stems preinoculated with *Au. pullulans*. The above procedure was repeated, substituting *Cl. rosea* CP63E for *Au. pullulans* AuP12, and incubating stems at 10°C instead of 12°C.

Mycoparasitism of *As. rabiei* and other fungi by *Cl. rosea*

Slide cultures were produced using glass microscope slides with a thin layer of malt extract agar (MEA). Three such cultures were established for each pair of isolates: CP63E (*Cl. rosea*) vs. AR19 (*As. rabiei*) and CP98B (*Cl. rosea*) vs. AR19. Slides, incubated 8–11 days in chambers over moistened filter paper, were stained with lactic acid–aniline blue (0.1%), and examined at 200–1000×. The procedure was repeated, substituting *Au. pullulans* AuP26 for AR19. We also reproduced the procedure five times, by pairing with each of the *Cl. rosea* isolates the first isolate for each of the last five species in Table 1. Additionally, the first isolate listed for each of those five species in Table 1, plus AuP12, AR19 and AR628, were paired with *Cl. rosea* CP63E and CP98B on culture plates of MEA. In each instance, the test isolate was inoculated to the centre of the culture dish of MEA, with CP63E inoculated to one side and CP98B to the other. Plates were incubated under ambient laboratory conditions until colonies made contact, at which time they were examined for reaction zones. The experiment was replicated.

Table 2
Pairings of *Didymella rabiei* isolates for production of ascospores

Nine outcrosses
AR20e (*MAT1-2*) × AR630 (*MAT1-1*)
AR20e (*MAT1-2*) × AR21d (*MAT1-1*)
AR20e (*MAT1-2*) × AR462 (*MAT1-1*)
AR460 (*MAT1-2*) × AR630 (*MAT1-1*)
AR460 (*MAT1-2*) × AR21d (*MAT1-1*)
AR460 (*MAT1-2*) × AR462 (*MAT1-1*)
AR428 (*MAT1-2*) × AR630 (*MAT1-1*)
AR428 (*MAT1-2*) × AR21d (*MAT1-1*)
AR428 (*MAT1-2*) × AR462 (*MAT1-1*)
Two self-'crosses'
AR20e × AR20e
AR21d × AR21d

Results

Floristic analysis

Fungi recovered from chickpea stems and debris in 2003 and 2004 are listed in Table 3. In order of relative abundance, the most frequently isolated fungi were *Al. tenuissima*, *Al. infectoria*, *U. consortiale*, *Epicoccum*

Table 3
Most abundant fungal colonists of chickpea stems

Species	Total percentage 2003[a]	2004[b]
Alternaria tenuissima (Kunze ex Pers.) Wilts.	36	35
Alternaria infectoria E.G. Simmons	12	20
Alternaria malorum (Ruehle) U. Braun, Crous & Dugan	4	0
Ulocladium consortiale (Thüm.) E.G. Simmons	12	6
Ulocladium atrum G. Preuss	2	6
Epicoccum purpurascens Ehrenb. ex Schlecht.	6	4
Fusarium pseudograminearum O'Donnell & T. Aoki	5	2
Fusarium acuminatum Ellis & Everh.	0	3
Fusarium solani (Mart.) Sacc.	1	4
Fusarium spp.	1	2
Unidentified[c]	6	3
Cladosporium herbarum (Pers.:Fr.) Link	3	2
Cladosporium cladosporioides (Fresen.) G.A. de Vries	0	2
Cladosporium sp.	1	< 1
Phoma-like spp.	3	4
Aureobasidium pullulans (de Bary) G. Arnauld	2	3
Clonostachys rosea (Link:Fr.) Schroers et al.	2	0
Botrytis cinerea Pers.:Fr.	0	1
Alternaria sp.	< 1	< 1
Ascochyta sp.	0	< 1
Botryotrichum keratinophilum Kushwaha & S.C. Agarwal	< 1	0
Curvularia inaequalis (Shear) Boedijn	< 1	0
Chaetomium sp.	0	< 1
Cladosporium macrocarpum G. Preuss	0	< 1
Fusarium oxysporum Schlechtend.:Fr.	0	< 1
Humicola sp.	< 1	< 1
Mucor sp.	0	< 1
Pithomyces chartarum (Berk. & M.A. Curtis) M.B. Ellis	< 1	0

[a]265 isolates from chickpea stems at harvest.
[b]358 isolates from chickpea stems 2 weeks postharvest.
[c]Non-sporulating, becoming non-viable, or otherwise unidentified.

Table 4
Mean radial growth (mm) of *Aureobasdium pullulans* and *Ascochyta rabiei* along autoclaved chickpea stems at 15 and 20°C by species, strain and RH level

	Growth (species)			Growth (strain)		
Species	15°C	20°C	Strain	15°C	20°C	RH*
Au. pullulans	18.63^{A}	28.42^{A}	AuP26	18.75^{Aa}	35.28^{Aa}	High
			AuP12	18.50^{Ba}	21.56^{Bb}	High
As. rabiei	14.19^{B}	16.48^{B}	AR628	14.25^{Bb}	17.28^{Bb}	High
			AR19	14.13^{Bb}	15.69^{Bb}	High
Au. pullulans	8.38^{A}	22.20^{A}	AuP26	8.75^{Aa}	25.25^{Aa}	Low
			AuP12	8.00^{Aa}	19.15^{ABa}	Low
As. rabiei	6.50^{B}	12.28^{B}	AR628	7.38^{Aa}	12.83^{Bb}	Low
			AR19	5.63^{Bb}	11.74^{Bb}	Low

*Relative humidity (RH) high, ≥90% RH; RH low, ≥80% RH. Within each RH level, means with the same upper case letter do not differ at $P \leq 0.05$ (LSD). Within each RH level, means with the same lower case letter do not differ at $P \leq 0.10$ (LSD; $N = 32$).

purpurascens and *U. atrum*. Also notable were other dematiaceous hyphomycetes including *Au. pullulans* and *Fusarium* species. *Clonostachys rosea* was isolated only in 2003.

Relative growth rates and interspecies growth inhibition

Relative growth on chickpea stems Growth at 15°C was less than at 20°C for both *Au. pullulans* and *As. rabiei*, and growth at lower humidity (RH ≥ 80%) was less than at higher humidity (RH ≥ 90%; Table 4). When data were analysed by species, *Au. pullulans* always grew more quickly than *As. rabiei*. When analysed by strain, both *Au. pullulans* strains grew more quickly than the *As. rabiei* strains, but in some instances differences were not significant (Table 4). When *Cl. rosea* was paired with *As. rabiei* (at 20°C and RH ≥ 90%) mean growth rates did not differ at $P \leq 0.10$ on the basis of species assignment. With regard to strain, the fastest mean growth (20.03 mm) was from *Cl. rosea* CP63E and the slowest (13.88 mm) was from *Cl. rosea* CP98B, with the two *As. rabiei* isolates being intermediate (data not shown).

Interspecies growth inhibition by As. rabiei and Au. pullulans on chickpea stems At 20°C and RH ≥ 90%, and at 15°C and RH ≥ 90%, *As. rabiei* and *Au. pullulans* colonies on the stems grew steadily until contacting the colony of the other species. Colonies did not merge and a reaction zone was apparent (Fig. 2). The distinctive fruiting bodies of each fungus were never observed to intermingle. In every chamber maintained at the lower humidity (RH ≥ 80%), growth slowed dramatically and sometimes ceased well prior to contacting the colony of the other species. In stems inoculated in the centre with a single strain of either species and not inoculated distally (Fig. 1), growth of both species slowed dramatically and sometimes ceased prior to reaching the ends of the stem in chambers with RH ≥ 80%. In each case from the experiments at 20°C + RH ≥ 90% and 15°C + RH ≥ 90%, when stem ends inoculated with *Au. pullulans* were excised and plated to agar, only *Au. pullulans* grew from the stem ends. On the controls (excised ends of stems inoculated in the centre with a single fungus), stems inoculated with *As. rabiei* produced only *As. rabiei* colonies, and stems inoculated with *Au. pullulans* produced only *Au. pullulans* colonies. Prior colonization of the distal portion of the stem by *Au. pullulans* effectively excluded growth of *As. rabiei* originating from the stem centre (Fig. 2), at least for the time frame during which the experiment was conducted. In one chamber at 20°C + RH ≥ 90%, one stem of the controls, supposedly inoculated with *As. rabiei*, was discarded because of contamination or erroneous inoculation with *Au. pullulans*.

The faster growth of *Au. pullulans* relative to that of *As. rabiei* was analogously documented on two agar media, one made with MEA and the other with pulverized chickpea seeds, pods and stems. At both 15 and 20°C strains of *Au. pullulans* grew more rapidly on MEA than strains of *As. rabiei* ($P < 0.001$, data not shown). On the chickpea medium, we also compared growth rates of *As. rabiei* with rates of *Al. infectoria*, *Al. tenuissima*, *Stemphylium* sp., *U. consortiale*, *E. purpurascens* and *Cl. rosea*, all of which grew faster than *As. rabiei* ($P < 0.01$, data not shown). When *As. rabiei* was paired with other species, inhibition zones were apparent in all crosses, except those involving *Cl. rosea*, in which cases *Cl. rosea* overgrew colonies of *As. rabiei*.

Inhibition by *Au. pullulans* of ascospores and conidia production in *D. rabiei*

The following crosses produced ascospores from four of four attempted crosses in the positive controls: AR630 × AR20e, AR462 × AR20e, AR630 × AR460, AR462 × AR460. The crosses AR462 × AR428 and AR421d × AR460 yielded ascospores from three of four and one of four crosses respectively. The crosses AR21d × AR20e, AR630 × AR428 and AR21d × AR428 did not produce ascospores in the positive controls, and were not further used in the analysis. Neither self-cross (AR20e, AR21d) produced ascospores, no ascospores were produced on any stems receiving prior treatment with *Au. pullulans*, nor were pycnidia seen to mature on any such stems. Hence, the suppression of sexual reproduction of *D. rabiei* by prior inoculation with *Au. pullulans* is regarded as total amongst all *D. rabiei* crosses producing ascospores in the positive controls, and the suppression of asexual reproduction of *D. rabiei* is similarly regarded as total in all instances in which stems were preinoculated with *Au. pullulans*. The relative abundance of ascospores produced in the positive controls varied. In most instances, more than 100 ascospores were produced on a given agar plate, but one cross of AR462 × AR20e, one of AR630 × AR460 and one of AR21d × AR460 produced fewer than 50 ascospores.

Clonostachys rosea was also effective for inhibiting reproduction of *D. rabiei*. In the positive controls, five of the nine attempted crosses produced ascospores

(AR460 × AR630 in two of four plates, AR20e × AR630 in one of four plates, AR20e × AR462 in three of four plates, AR428 × AR462 in one of four plates and AR460 × AR462 in one of four plates). Numbers of ascospores were not quantified beyond 'numerous' vs. 'absent'. In no instance did the stems receiving prior inoculation with *Cl. rosea* produce any ascospores. Self-crosses produced no ascospores. In no instances did stems receiving prior inoculation with *Cl. rosea* produce mature pycnidia. Prior inoculation with *Cl. rosea*, as with *Au. pullulans*, completely suppressed both sexual and asexual reproduction by *D. rabiei*.

Mycoparasitism of *D. rabiei* and other fungi by *Cl. rosea*

Both CP63E and CP98B (*Cl. rosea*) formed appressoria on hyphae of AR19 *D. rabiei*, and subsequently penetrated hyphae of AR19 and grew internally (Figs 3 and 4). Interactions of *Cl. rosea* with non-target fungi varied. *Aureobasidium pullulans* hyphae tended to grow deeper into the medium while *Cl. rosea* occupied the agar surface, with hyphae of *Cl. rosea* eventually contacting hyphae of *Au. pullulans* and branching prior to prolonged contact. Hyphae of *Cl. rosea* formed profuse coils and appressoria on hyphae of *Al. infectoria*, *Al. tenuissima*, *Stemphylium* sp. and *U. consortiale*. Interactions with *E. purpurascens* were similar to those with *Au. pullulans*. Penetration of hyphae of the other fungus by *Cl. rosea* was observed only when *Cl. rosea* was paired with *D. rabiei*.

When paired on MEA plates, colonies of *D. rabiei* were in all instances overgrown by the strains of *Cl. rosea*, which sporulated on them. There was no evidence of a reaction zone between the two taxa. When paired with other taxa (*Au. pullulans* and the last five species in Table 1) on MEA, *Cl. rosea* was uninhibited. Colonies of *Cl. rosea* overgrew, and to a limited extent sporulated on, the surface of colonies of the taxa with which they were paired. No reaction zones were apparent between *Cl. rosea* and the other taxa, although in the areas of overlap between *Cl. rosea* and the colonies of other taxa, *Cl. rosea* sporulated less prolifically than normal.

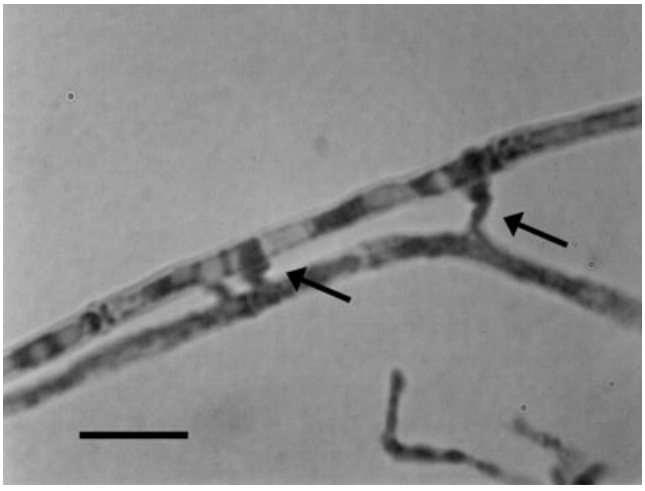

Fig. 3 *Clonostachys rosea* CP98B forms appressoria (arrows) on hypha of *Didymella rabiei* AR19 (bright field, bar = 10 μm)

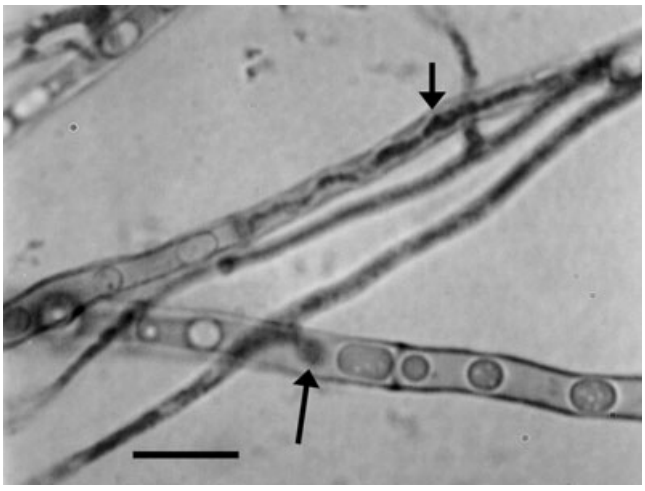

Fig. 4 *Clonostachys rosea* CP63E forms appressorium (long arrow) on hypha of *Didymella rabiei* AR19, and grows internally in an adjacent hypha (short arrow; bright field, bar = 10 μm)

Discussion

The most frequently recovered fungal colonists of chickpea debris were common dematiaceous hyphomycetes, most of which have been reported in analogous surveys from other plant substrata (e.g. Dugan and Roberts, 1994; Dugan and Lupien, 2002; Dugan et al., 2002). Conspicuous by its absence in the 2003 survey was *Al. alternata* (sensu Simmons, 1990, 1995), although *Al. tenuissima* and *Al. infectoria*, two common species, were readily recovered, as was the less widely reported *Al. malorum*, recently reassigned to that genus from *Cladosporium* (Braun et al., 2003). Also conspicuously absent in 2003–2004 were representatives of *Stemphylium* (teleomorphs in *Pleospora*). Both *Stemphylium* sp. and *Pleospora* sp. had been encountered in prior inspections of chickpea residue (W. Kaiser and T. Peever, unpublished data), as was *Au. pullulans* (F. Dugan, unpublished data). We account for the repeated isolation of *F. pseudograminearum* (*F. graminearum* group 1) and *F. acuminatum* by the prevalence of cereals and grasses in the immediate vicinity of our plots and in rotations with chickpea, by occasional grassy weeds, by the persistence of *F. graminearum* in residues (Pereya et al., 2004) and by the wide range of plant substrata from which these species have been documented (Ginns, 1986; Farr et al., 1989). *Fusarium pseudograminearum* is commonly isolated from stems of wheat and barley in eastern Washington (T. Paulitz, personal communication).

Amongst those taxa recovered by us from chickpea debris and previously documented as experimental biological control agents against phytopathogens are *Au. pullulans*, *E. purpurascens* and *U. atrum* (e.g. Wittig et al., 1997; Kessel et al., 1999; Castoria et al., 2001). *Clonostachys rosea* has also been used in numer-

ous experiments in biological control (Xue, 2003a,b), including work on chickpea (Burgess et al., 1997; Prasad and Rangeshwaran, 1999). *Clonostachys* species other than *Cl. rosea* have also evoked interest for their potential in biological control (Evans et al., 2003).

Many fungi have been employed as biocontrol agents against phytopathogenic fungi (Dugan, 1996), including phytopathogens that are relatives of *As. rabiei* (Pfender, 1988; Pfender et al., 1993; Ouimet et al., 1997; Philion et al., 1997; Carisse et al., 2000; Bujold et al., 2001; Carisse and Bernier, 2002). We isolated and identified to species none of the fungi utilized and proven effective by these other authors against relatives of *As. rabiei*, with the significant exception of *E. purpurascens*, but we note that isolates denoted as *Aureobasidium* sp. were sometimes utilized by these other workers (Ouimet et al., 1997; Philion et al., 1997).

Candidate organisms for biological control of phytopathogenic fungi should meet the following criteria: (i) well established in nature in the geographical area in which they are to be deployed, (ii) easy to grow in mass fermentation culture, (iii) survive well on organic debris, (iv) non-pathogenic to plants, humans and animals (i.e. should be Biosafety Level 1), (v) non-allergenic to humans and animals and (vi) demonstrated ability to antagonize pertinent pathogens (Dugan, 1996; Goettel et al., 2001). *Aureobasidium pullulans* conforms well to the above criteria (Solomon and Jelks, 1997; Andrews et al., 2002), is well represented in the microflora of chickpea debris, has been the subject of extensive use in other experimental biological control (e.g. Leibinger et al., 1997; Lima et al., 1997; Dik and Elad, 1999; Dik et al., 1999; Schena et al., 1999, 2002; Ippolito et al., 2000; Castoria et al., 2001), and also produces pullulan, a natural adhesive that is used as a food additive (Ducrey et al., 1992) and which helps retain spores on surfaces (Bardage and Bjurman, 1998).

Potential allergenicity is a prime safety concern for fungal biological control agents (Butt and Copping, 2000). *Alternaria*, *Ulocladium* and *Stemphylium* species produce the Alt-1 allergen (and other allergens as well) and impact large sectors of the human population (Agarwal et al., 1982; Lelong et al., 1986; Horner et al., 1995; Day and Ellis, 2001). *Epicoccum purpurascens* and *Cladosporium* species are also important allergens for major sectors of the population (Day and Ellis, 2001; Bisht et al., 2004). Approximately 20% of the total human population are atopic and readily sensitized to spore concentrations of $10^6/m^3$ in the ambient air (Lacey, 1981). *Aureobasidium pullulans* is less allergenic than the above species, affecting approximately 8–12% of the human population experiencing fungal allergy (Solomon and Jelks, 1997). Like virtually all common fungi that are Biosafety Level 1, *Au. pullulans* has occasionally been isolated from puncture wounds or from immunocompromised patients in clinical settings (de Hoog et al., 2000). We failed to locate any literature implicating *Clonostachys* with allergenic or pathogenic activity in humans or animals. We realize that massive release of mycoparasitic *Cl. rosea* into the environment may have implications for non-target fungi, hence inclusion of the latter into our experiments. Unlike Xue (2003a) we observed no penetration of hyphae of *Alternaria*, but we did observe appressoria of *Cl. rosea* in these interactions. In other respects, our results were similar to those of Xue (2003a), i.e. *Cl. rosea* coiled around hyphae of other fungi.

Based on the above criteria, and considering the ability of our strains of *Au. pullulans* to grow faster than *D. rabiei*, to exclude it from substrate already occupied by *Au. pullulans*, and to completely suppress sexual and asexual reproduction under our experimental conditions, we believe that *Au. pullulans* has potential as a biological control agent against Ascochyta blight of chickpea. Based on its abilities as a mycoparasite, *Cl. rosea* may also be a prospective agent because it is mycoparasitic on *D. rabiei*, successfully suppressed sexual and asexual reproduction in our laboratory, and is neither known for pathogenicity nor allergenicity to humans or animals. We have initiated small-scale field trials using *Au. pullulans* and *Cl. rosea* on chickpea debris infested with *D. rabiei* to determine if the sexual stage can be suppressed by these agents under natural conditions.

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References

Agarwal MK, Jones RT, Yunginger JW. (1982) Shared allergenic and antigenic determinants in *Alternaria* and *Stemphylium* extracts. *J Allergy Clin Immunol* **70**:437–444.

Andersen B, Thrane U. (1996) Differentiation of *Alternaria infectoria* and *Alternaria alternata* based on morphology, metabolite profiles, and cultural characteristics. *Can J Microbiol* **42**:685–689.

Andrews JH, Spear RN, Nordheim EV. (2002) Population biology of *Aureobasidium pullulans* on apple leaf surfaces. *Can J Microbiol* **48**:500–513.

Aoki T, O'Donnell K. (1999) Morphological and molecular characterization of *Fusarium pseudograminearum* sp. nov., formerly recognized as the group 1 population of *F. graminearum*. *Mycologia* **91**:597–609.

Bardage S, Bjurman J. (1998) Isolation of an *Aureobasidium pullulans* polysaccharide that promotes adhesion of blastospores to waterborne paints. *Can J Microbiol* **44**:954–958.

Barve MP, Arie T, Salimath S, Muehlbauer FJ, Peever TL. (2003) Cloning and characterization of the mating type (MAT) locus from *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) and a MAT phylogeny of legume-associated *Ascochyta* spp. *Fungal Genet Biol* **39**:151–167.

Bisht V, Arora N, Singh BP, Gaur SN, Sridhara S. (2004) Purification and characterization of a major cross-reactive allergen from *Epicoccum purpurascens*. *Int Arch Allergy Immunol* **133**:217–224.

Braun U, Crous PW, Dugan F, Groenewald JZ, de Hoog GS. (2003) Phylogeny and taxonomy of *Cladosporium*-like hyphomycetes, including *Davidiella* gen. nov., the teleomorph of *Cladosporium* s. str. *Mycol Prog* **2**:3–18.

Bujold I, Paulitz TC, Carisse O. (2001) Effect of *Microsphaeropsis* sp. on the production of perithecia and ascospores of *Gibberella zeae*. *Plant Dis* **85**:977–984.

Burgess DR, Bretag T, Keene PJ. (1997) Biocontrol of seedborne *Botrytis cinerea* in chickpea with *Gliocladium roseum*. *Plant Pathol* **46**:298–305.

Butt TM, Copping LG. (2000) Fungal biological control agents. *Pestic Outlook 2000* **5**:186–191.

Carisse O, Bernier J. (2002) *Microsphaeropsis ochracea* sp. nov associated with dead apple leaves. *Mycologia* **94**:297–301.

Carisse O, Philion V, Rolland D. (2000) Effect of fall application of fungal antagonists on spring ascospre production of the apple scab pathogen, *Venturia inaequalis*. *Phytopathology* **90**:31–37.

Castoria R, DeCurtis F, Lima G, Caputo L, Pacifico S, DeCicco V. (2001) *Aureobasidium pullulans* (LS-30) an antagonist of postharvest pathogens of fruits: study on its modes of action. *Postharvest Biol Technol* **22**:7–17.

Day JH, Ellis AK. Allergenic microorganisms and hypersensitivity. In: Flannigan B, Samson RA, Miller JD (eds), *Microorganisms in Home and Indoor Work Environments*, London, UK, Taylor & Francis, 2001, pp. 103–127.

Dik AJ, Elad Y. (1999) Comparison of antagonists of *Botrytis cinerea* in greenhouse-grown cucumber and tomato under different climatic conditions. *Eur J Plant Pathol* **105**:123–137.

Dik AJ, Koning G, Köhl J. (1999) Evaluation of microbial antagonists for biological control of *Botrytis cinerea* stem infection in cucumber and tomato. *Eur J Plant Pathol* **105**:115–122.

Ducrey SLM, Sineriz F, Castro-Guillermo R. (1992) A specrophotometric method for the quantitative measurement of pullulan. *J Microbiol Methods* **16**:253–258.

Dugan FM. Biological pest control. In: *1997 McGraw-Hill Yearbook of Science and Technology*, New York, USA, McGraw-Hill, 1996, pp. 45–47.

Dugan FM, Lupien SL. (2002) Filamentous fungi quiescent in seeds and culm nodes of weedy and forage grass species endemic to the Palouse region of Washington and Idaho. *Mycopathologia* **156**:31–40.

Dugan FM, Roberts RG. (1994) Etiology of pre-harvest infections of Bing cherry fruits by fungi. *Phytopathology* **84**:1031–1036.

Dugan FM, Lupien SL, Grove GG. (2002) Incidence, aggressiveness, and *in planta* interactions of *Botrytis cinerea* and other filamentous fungi quiescent in grape berries and dormant buds in central Washington state. *J Phytopathol* **150**:375–381.

Ellis MB. *Dematiaceous Hyphomycetes*. Kew, Surrey, England, Commonwealth Mycological Institute, 1971.

Ellis MB. *More Dematiaceous Hyphomycetes*. Kew, Surrey, England, Commonwealth Mycological Institute, 1976.

Evans HC, Holmes KA, Thomas SE. (2003) Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri*, in Ecuador and a preliminary assessment of their potential as biocontrol agents of cocoa diseases. *Mycol Prog* **2**:149–160.

Farr DF, Bills GF, Chamuris GP, Rossman AY. *Fungi on Plants and Plant Products in the United States*. St Paul, Minnesota, USA, APS Press, 1989.

Ginns JH. *Compendium of Plant Disease and Decay Fungi in Canada, 1960–1980*. Canada, Agriculture Canada Publication 1813, 1986.

Goettel MS, Hajek AE, Siegel JP, Evans HC. Safety of fungal biocontrol agents. In: Butt TM, Jackson, Magan N (eds), *Fungi as Biocontrol Agents*, Wallingford, UK, CABI Pub., 2001, pp. 347–375.

Hermanides-Nijhof EJ. (1977) *Aureobasidium* and allied genera. *Stud Mycol* **15**:141–177.

Ho M-HM, Castañeda RF, Dugan FM, Jong SC. (1999) *Cladosporium* and *Cladophialophora* in culture: descriptions and an expanded key. *Mycotaxon* **72**:115–157.

de Hoog GS, Yurlova NA. (1994) Conidiogenesis, nutritional physiology and taxonomy of *Aureobasidium* and *Hormonema*. *Antonie Van Leeuwenhoek* **65**:41–54.

de Hoog GS, Guarro J, Gené J, Figueras MJ. *Atlas of Clinical Fungi*, 2nd edn. Utrecht, the Netherlands and Universitat Rovira i Virgili, Rues, Centraalbureau voor Schimmelcultures, 2000.

Horner WE, Helbling A, Salvaggio JE, Lehrer SB. (1995) Fungal allergens. *Clin Microbiol Rev* **8**:161–179.

Ippolito A, El Ghaouth A, Wilson CL, Wisniewski M. (2000) Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of postharvest responses. *Postharvest Biol Technol* **19**:265–272.

Kaiser WJ. Epidemiology of *Ascochyta rabiei*. In: Singh KB, Saxena MC (eds), *Disease Resistance Breeding in Chickpea*, Aleppo, Syria, ICARDA, 1992.

Kaiser WJ, Hannan RM. (1987) Survival of *Ascochyta rabiei* in chickpea debris. *Phytopathology* **77**:1240.

Kessel GJT, de Haas BH, Lombaers-van der Plas CH et al. (1999) Quantification of mycelium of *Botrytis* spp. and the antagonist *Ulocladium atrum* in necrotic leaf tissue of cyclamen and lily by fluorescence microscopy and image analysis. *Phytopathology* **89**:868–876.

Kushwaha RKS, Agrawal SC. (1976) A new keratinophilic species of *Botryotrichum*. *Trans Mycol Soc Jpn* **17**:18–22.

Lacey J. The aerobiology of conidial fungi. In: Cole GT, Kendrick B (eds), *Biology of Conidial Fungi*, New York, USA, Academic Press, 1981, pp. 373–416.

Leibinger WL, Breuker B, Hahn M, Mengen K. (1997) Control of postharvest pathogens and colonization of the apple surface by antagonistic microorganisms in the field. *Phytopathology* **87**:1103–1110.

Lelong M, Henard J, Wattre P, Duprey J, Thelliez P, Miersman R. (1986) Does immediate-type respiratory allergy occur regarding *Stemphylium*? Evaluation of 39 challenge tests. *Allerg Immunol (Paris)* **18**:21**,** 23, 25–26.

Lima TK, Ippolito A, Nigro F, Salerno M. (1997) Effectiveness of *Aureobasidium pullulans* and *Candida oleophila* against postharvest strawberry rots. *Postharvest Biol Technol* **10**:169–178.

Nelson PE, Toussoun TA, Marassas WFO. *Fusarium species: An Illustrated Manual for Identification*. University Park, Pennsylvania, USA, Pennsylvania State University Press, 1983.

Ouimet A, Carisse O, Neuman P. (1997) Environmental and nutritional factors affecting the in vitro inhibition of the vegetative growth of *Venturia inaequalis* by five antagonistic fungi. *Can J Bot* **75**:632–639.

Peever TL, Salimath SS, Su G, Kaiser WJ, Muehlbauer FJ. (2004) Historical and contemporary multilocus population structure of *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) in the Pacific Northwest of the United States. *Mol Ecol* **13**:291–309.

Pereya SA, Dill-Macky R, Sims AL. (2004) Survival and inoculum production of *Gibberella zeae* in wheat residue. *Plant Dis* **88**:724–730.

Pfender WF. (1988) Suppression of ascocarp formation in *Pyrenophora tritici-repentis* by *Limonomyces roseipellis*, a basidiomycete from reduced-tillage wheat straw. *Phytopathology* **78**:1245–1258.

Pfender WF, Zhang W, Nus A. (1993) Biological-control to reduce inoculum of the tan spot pathogen *Pyrenophora tritici-repentis* in surface-borne residues of wheat fields. *Phytopathology* **83**:371–375.

Philion V, Carisse O, Paulitz T. (1997) In vitro evaluation of fungal isolates for their ability to influence leaf rheology, production of pseudothecia, and ascospores of *Venturia inaequalis*. *Eur J Plant Pathol* **103**:441–452.

Prasad RD, Rangeshwaran R. (1999) Granular formulation of *Trichoderma* and *Gliogladium* spp. in biocontrol of *Rhizoctonia solani* of chickpea. *J Mycol Plant Pathol* **29**:222–226.

dos Santos MA, Muchovej JJ, Ferraz S. (1993) A new species of *Botryotrichum* parasitic on nematode eggs. *Mycotaxon* **48**:271–274.

Schena L, Ippolito A, Zehavi T, Cohen L, Nigro F, Droby S. (1999) Genetic diversity and biocontrol activity of *Aureobasidium pullulans* isolates against postharvest rots. *Postharvest Biol Technol* **17**:189–199.

Schena L, Sialer MF, Gallitelli D. (2002) Molecular detection of strain L47 of *Aureobasidium pullulans*, a biocontrol agent of postharvest diseases. *Plant Dis* **86**:54–60.

Schroers H-J. (2001) A monograph of *Bionectria* (Ascomycota, Hypocreales, Bionectriaceae) and its *Clonostachys* anamorphs. *Stud Mycol* **46**:1–214.

Seifert KA. (1996) *FusKey: Fusarium* Interactive Key. Agriculture and Agri-Food Canada. (This reference is no longer available on an active website. The complete reference can be obtained from the author, K. Seifert.)

Simmons EG. (1967) Typification of *Alternaria*, *Stemphylium* and *Ulocladium*. *Mycologia* **59**:67–92.

Simmons EG. (1986) *Alternaria* themes and variations (22–26). *Mycotaxon* **25**:287–308.

Simmons EG. (1990) *Alternaria* themes and variations (27–53). *Mycotaxon* **37**:79–119.

Simmons EG. (1995) *Alternaria* themes and variations (112–144). *Mycotaxon* **60**:55–163.

Solomon WR, Jelks ML. Airborne allergens. In: Kaplan AP (ed.), *Allergy*, 2nd edn, Philadelphia, USA, W.B. Saunders Co., 1997, pp. 344–385.

Stevens RB. (ed.) *Mycology Guidebook*. Seattle, USA, University of Washington Press, 1981, pp. 665.

Trapero-Casas A, Kaiser WJ. (1992a) Influence of temperature, wetness period, plant age, and inoculum concentration on infection and development of Ascochyta blight of chickpea. *Phytopathology* **82**:589–596.

Trapero-Casas A, Kaiser WJ. (1992b) Development of *Didymella rabiei*, the teleomorph of *Ascochyta rabiei*, on chickpea straw. *Phytopathology* **82**:1261–1266.

Trapero-Casas A, Navas-Cortes JA, Jimenez-Diaz RM. (1996) Airborne ascospores of *Didymella rabiei* as a major source of primary inoculum for Ascochyta blight epidemics in chickpea crops in southern Spain. *Eur J Plant Pathol* **102**:237–245.

Wilson AD, Kaiser WJ. (1995) Cytology and genetics of sexual compatibility in *Didymella rabiei*. *Mycologia* **87**:795–804.

Wittig HPP, Johnson KB, Pscheidt JW. (1997) Effect of epiphytic fungi on brown rot, blossom blight and latent infections in sweet cherry. *Plant Dis* **81**:382–387.

Xue AG. (2003a) Biological control of pathogens causing root rot complex in field pea using *Clonostachys rosea* strain ACM941. *Phytopathology* **93**:329–335.

Xue AG. (2003b) Efficacy of *Clonostachys rosea* strain ACM941 and fungicide seed treatments for controlling the root rot complex of field pea. *Can J Plant Sci* **83**:519–524.

Yurlova NA, de Hoog GS, Gerrits van Ende AHG. (1999) Taxonomy of *Aureobasidium* and allied genera. *Stud Mycol* **43**:63–69.